Research Article

Role of QS Genes in QS-Dependent Virulence Factors and the Inhibitory Effect of Clove Oil

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Abstract | Pseudomonas aeruginosa is a Gram-negative bacteria found in various environments, including plants, soil, and water. It is a significant opportunistic pathogen in humans, particularly in immune compromised individuals like cancer patients, severely burned patients, and those with cystic fibrosis. The bacteria form biofilms, which attach to medical devices or host tissue to establish infection. Quorum sensing (QS) is a cell-to-cell communication mechanism that controls the formation of biofilms and the production of virulent factors. This study evaluated the association between quorum sensing and virulence in 30 samples collected from microbiology labs in Pakistan. All isolates showed maximum resistance to various antibiotics. Different virulence factors, such as biofilm production, pyocyanin, and twitching motility, were phenotypically detected. Clove oil was found to have an inhibitory effect on QS, which should be clinically exploited.

Received | March 13, 2025; **Accepted** | May 08, 2025; **Published** | August 04, 2025
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Citation | Siddique, S., M. Farooq and I. Siddique. 2025. Role of QS genes in QS-dependent virulence factors and the inhibitory effect of clove oil. *Sarhad Journal of Agriculture,* 41(3): 1241-1248.
DOI | https://dx.doi.org/10.17582/journal.sja/2025/41.3.1241.1248
Keywords | Antibiotics, Bacteria, PCR, Tetracycline, Clove oil, Gentamycine

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Introduction

Pseudomonas aeruginosa is a Gram-negative bacterium found in various habitats, including soil, water, and plants. It can transform into an opportunistic pathogen when host tissue barriers are breached, or the immune system is suppressed. Pseudomonal infections are responsible for 10%-20% of nosocomial infections, such as septicemia, cystic fibrosis, burns, and wound infections. The severity of Pseudomonal infections is due to the production of virulent factors such as pyocyanin, flagella, and pili. Biofilm formation is crucial for the establishment of *P. aeruginosa* infections on host tissues and medical devices, which can lead to antibiotic resistance (Han *et al.*, 2022). Another virulence factor produced by these bacteria is the blue redox-active exoproduct pyocyanin, which can easily penetrate biological membranes and cause neutrophil apoptosis. Cell-associated virulence factors include type IV pili, which facilitate adherence to eukaryotic cell surfaces and twitching motility. Pseudomonas elastase, also known as pseudo lysin, is a key virulence factor expressed by this bacterium. This metalloprotease degrades host tissue proteins

and key bio molecules involved in innate immunity. Cystic fibrosis, a hereditary disease, affects sweat glands, hepatobiliar system, reproductive tracts, lungs, and pancreas. Quorum sensing (QS) is a cell-to-cell communication process that enables bacteria to sense their population density and organize gene expression accordingly. Through QS, bacteria organize activities such as plasmid conjugation, bioluminescence, and the production of different virulence factors (Raposo *et al*., 2017).

Quorum sensing (QS) is a mechanism in which Gram positive and Gram-negative bacteria favor different signaling components. The term QS was first proposed by J. Woodland Hastings and Kenneth Nealson two decades ago, based on the bacterial population density system observed in various microbes, including *P. aeruginosa*, *Erwinia carotovora, Agrobacterium tumefaciens*, and *Vibrio fischeri* (Jingjing *et al*., 2019). The main mechanism involves the expression and release of signal molecules to the environment by bacterial cells within the population. As the number of cells increases, the level of signal molecules increases until the microorganism senses that a threshold has been reached, and gene activation or repression or depression occurs through the activity of sensor regulator mechanisms (Van Delden and Iglewski, 1998). The idea of QS in *P. aeruginosa* was based on the study on prototype luxR and luxI systems in *V. fischeri*. Las R was primarily recognized as the main mediator in the expression of las B gene translating metalloprotease elastase, and LasR was also considered a universal mediator in regulating virulence genes in *P. aeruginosa*. LuxI in *P. aeruginosa* was suggested to synthesize AHL signals with elastase-mediating and auto-inducing characteristics (Fuqua *et al*., 1994).

Several studies have confirmed the contribution of quorum sensing in disease-causing of *P. aeruginosa*. Virulence factors expressed by this bacterium are regulated by the QS system, such as the production of rhamnolipid and pyocyanin, and Las A protease and Las B elastase activities are controlled by the las system. These proteases enhance replication of the bacteria within the host by interrupting with the immune system. The two distinct QS systems in *P. aeruginosa*, rhl and las, depend on N-acyl homoserine lactone (AHL) signal molecules, also called auto inducers (Wang *et al*., 2018). This sophisticated signaling mediates the expression of about 350 genes, with 30% encoded for virulence factors expression and biofilm formation. The significance of QS in the virulence of *P. aeruginosa* has been studied in multiple animal models, including mouse models of acute and chronic lung infections. Clove oil has anti-QS properties, and clove extracts may be useful as an anti-infective drug. However, secure, broad-spectrum, and stable quorum sensing inhibitors with proven therapeutic uses are still to be revealed and exploited (Farooq *et al*., 2024).

Materials and Methods

Sample collection

In this present study, about thirty samples were collected from multiple hospitals in Pakistan over the period from January 2020 to June 2021. Out of thirty, 10 strains were collected from wounds, 5 from sputum, 10 from urine samples and 5 from nasal discharge. For sample collection swab methods were used. The collected samples were immediately transported in sterile specimen containers to the laboratory.

Detection of isolates

The study used a conventional culture technique for *P. aeruginosa* isolation, using Pseudomonas CN Selective Agar for isolation and Cetrimide agar for subculturing and identification tests. Cetrimide is selective agent and inhibit microbial flora and enhance the production of *P. aeruginosa* pigments such as fluorescence and pyocyanin and fluorescence which show specific yellow green and blue-green color. Molecular confirmation of isolates targeting the species-specific oprL gene region was performed using PCR.

Identification of isolates

Species identification was conducted using a series of biochemical tests, including indole, methyl red, Simmons, citrate agar, catalase, and oxidase tests, in addition to evaluating culture characteristics, staining properties, and microscopic examination. Appearance of colonies, which were gained after 24 hours of incubation on Cetrimide agar plates were studied for morphological and cultural appearances, texture of colonies, color and shape were noted. Bacterial isolates were tested for catalase production by catalase test. Other tests were also mentioned. Cells were examined after gram staining.

Antimicrobial susceptibility testing via disk diffusion method

Antimicrobial sensitivity was carried out based on Kirby Bauer disc diffusion method. There was antibiotic that had a disc to check resistance or susceptibility of bacteria to that specific antibiotic. For this, bacteria were incubated for overnight at 37 degrees with antibiotics on Mueller Hinton agar. After incubation, some discs showed clear zones of bacterial growth. This was called zone of inhibition. If the size of inhibition was greater than or equal to standard size then specific antibiotics are supposed to be active for that specific bacteria. While some discs have no or little clear zone, which shows that specific bacteria was resistant to that antibiotic.

Biofilm assay: The biofilm-forming capability of the strains was evaluated using the polystyrene micro titer plate method reported by (O'Toole *et al.*, 2000) with slight alterations. *P. aeruginosa* were pre-cultured for overnight in nutrient broth. After incubation, micro titer plate was placed upside down and shaken to dump out cells and remove excess liquids. After drying, plate was photographed for qualitative assay.

Pyocyanin assay: *Pseudomonas aeruginosa* is the only gram-negative bacteria having the ability of producing the very distinct water-soluble pigment pyocyanin. This ability of isolates was identified by using King Agar (P Agar).

Twitching motility: To test the twitching ability of specimens, isolates were inoculated on to LB agar medium. The palates were then incubated at 37 °C overnight. After overnight incubation at 37^{0}C, plates were then left for 24-48 hrs at room temperature (< 25 ˚C).

Influence of clove oil on QS mediated virulence factors

The minimum inhibitory concentration (MIC) of clove oil was assessed against the 11 most virulent strains which were all twitching positive, biofilm forming, pyocyanin production and QS genes, by the broth micro dilution method (Smoglica *et al.*, 2023).

Biofilm inhibition assay: The MIC effects of clove oil on biofilm development were assessed by adding 12.8% clove oil to the wells of micro titer plate, clove-oil-free wells were used as controls. Then the assay was completed as explained earlier (Husain *et al.*, 2003).

Pyocyanin production inhibition assay

To check MIC influence of clove oil (12.8%) on pyocyanin production, clove oil was added during preparation of King A agar. After that, slants were prepared and were streaked while one slant was left untreated as a control. Then the slants were incubated for four to five days at 30–32 ˚C (Husain *et al.*, 2003).

Twitching motility inhibition assay

To check the effect of MIC of clove oil on twitching motility, isolates were cultured by being stabbed into LB agar plates provided with 12.8% clove oil using sterile toothpicks. Then the plates were incubated for 24hrs at 37˚C degrees and then left for a further 24-48 hrs at room temperature (< 25˚C).

Detection of QS genes by PCR

The chromosomal DNA was extracted by phenol chloroform method. Overnight bacterial broth culture was centrifuged for 5 min at 10,000 rpm. Supernatant was discarded while the pellet was dissolved in 200 µL of T-buffer in Eppendorf tubes. The tubes were then centrifuged at 10,000 rpm for 5 min, the supernatant was discarded again. 700 µL of 10% SDS was added to the pellet and dissolved well by repeated pipetting. Eppendorf tubes were then incubated for two hours at 65 °C (mixed after 1 hour inversion) and then cooled at room temperature. 700 µL of phenol: chloroform: isoamyl alcohol (25:25:1) was added, mixed by intensive shaking and centrifuged for 4 min at 12,000 rpm. The upper aqueous layer was shifted gently to a new Eppendorf tube without disrupting the middle layer. Phenol: chloroform: isoamyl alcohol and centrifugation step were repeated twice, and 100 µL of 3M sodium acetate (pH 4.8) was added to the supernatant followed by addition of 700 µL of chilled isopropanol. Mixture was homogenized gently by invasion and incubation at -20 degrees overnight. Tubes were centrifuged at 12,000 rpm for 4 min and supernatant was discarded. The pellet was washed with 70% ethanol and centrifuged for 4 min at 12.000. At the end pellet was then dried and dissolved in 50 µL T buffer on sterile Nuclease free water. The DNA was stored at -4 degrees until used.

Statistical analysis

The relationship between the presence of QS genes and the expression of virulence factors was evaluated using SPSS software and $p<0.05$ was considered statistically significant.

Results

Streaking was done on Cetrimide agar a selective media for *P. aeruginosa*. *P. aeruginosa* express several water-soluble iron chelators, including the yellow-brown and yellow-green, fluorescent pyoverdine. When the blue water soluble pyocyanin combines with pyoverdine combines, the bright green color specific of *Pseudomonas aeruginosa* was created. The presence of bacterial growth on the medium is suggestive of a positive reaction. Visual analysis may expose the distinctive blue to yellow green color which shows the expression of pyocyanin. Both fluorescein and pyocyanin are normally expressed by *P. aeruginosa* isolate.

Identification of P. aeruginosa through microscopy

Gram staining: Gram staining was carried out as a confirmatory test. Under microscopy red color rod shaped was observed and confirmed as *Pseudomonas aeruginosa* because it is gram negative, and rod shaped.

P. aeruginosa is a gram-negative rod-shaped bacterium as shown in Figure 1 under microscopy.

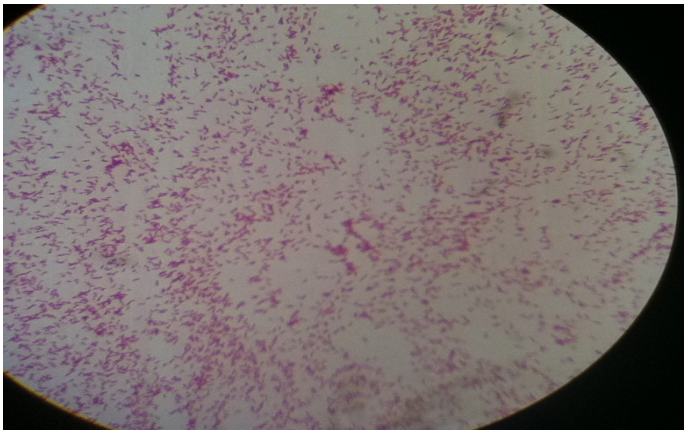

Figure 1: *Appearances of P. aeruginosa under microscopy.*

Biochemical characteristics

The isolates were subjected to biochemical tests including indole, Methyl red (MR), Simmon's citrate, catalase and oxidase tests.

Catalase test

This test was done that revealed that *P. aeruginosa* has catalase enzyme which degrade H_2O_2 and bubble formation occurred.

Citrate test

Citrate test shows that strains have the potency to use sodium citrate as its sole carbon source and inorganic ($NH^4H^2PO_4$) is only fixed nitrogen source. Blue color shows positive citrate consumption test and green color indicate negative results.

Indole test

An indole test was carried out to check out the potency of *P. aeruginosa* to break down the amino acid tryptophan to indole, which collects in the medium. There were no color changes after the addition of indole spot reagent because this bacterium does not possess tryptophanase enzyme. Tubes containing tryptophan broth were streaked with culture, incubated at 37 degrees overnight. Next day a few drops of indole spot reagent were added to tubes and observe color change.

Antibiotics sensitivity testing

Sensitivity testing was performed to different antibiotics by disc diffusion method and zone of inhibition was measured according to CLSI guidelines. Out of these thirty isolates most were resistant to the used antibiotics in Figure 2 and Table 1.

Table 1: *Antimicrobial susceptibility of isolates.*

Antibiotics	Resistant N (%)	Sensitive N (%)
Ampicillin	28(93)	2(6)
Amoxicillin	29(98)	1(2)
Augmentin	27(94)	3(3)
Cefadroxil	28(93)	2(6)
Sulbactam sodium	21(70)	9(30)
Cefazolin	29(98)	1(2)
Cefoxitine	28(96)	2(4)
Ceftriaxone	22(73)	8(26)
Cefoperazone	15(50)	15(50)
Cefepime	17(56)	13(43)
Meropenem	18(60)	12(40)
Co-trimoxazole	25(83)	5(16)
Tetracycline	19(63)	11(36)
Rifampicin	27(90)	3(10)
Chloramphenicol	24(80)	6(20)
Polymyxin	7(23)	23(76)
Nalidixic acid	12(40)	18(60)
Levofloxacin	13(43)	17(56)
Gentamycin	20(66)	10(33)

Biofilm assay

Biofilm forming capability of strains were detected in polystyrene micro titer as explained earlier. They were disseminated according to strength of biofilm forming ability. Biofilm production was assessed by micro titer

plate and revealed those 10 (33.33%) strong biofilms producers, 6 (20%) moderate producers, 14 (46.66%) were weak or non-biofilm former in Figure 4.

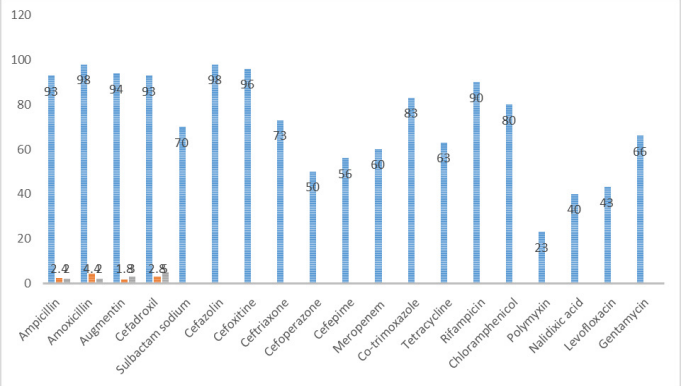

Figure 2: *Percentage resistance of isolation to different antibiotics.*

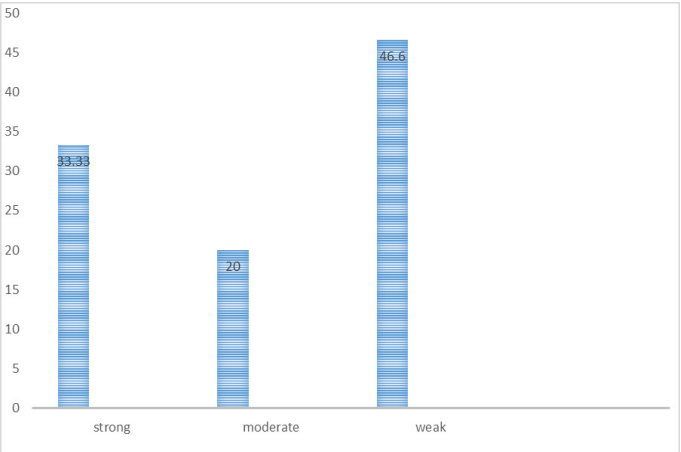

Figure 3: *Micro titer reader for quantifying Biofilm.*

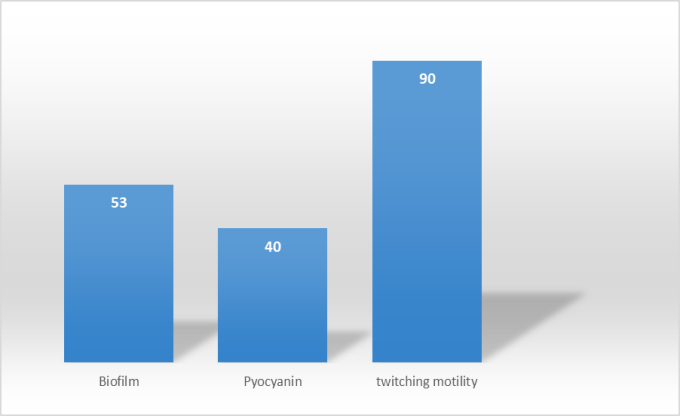

Figure 4: *Most isolates were positive for twitching motility followed by biofilm and pyocyanin production respectively.*

As Figure 3 shows that out of these thirty isolated, mostly were weak biofilm producers (46.66) followed by strong biofilm producers which count for about (33.3) and other remaining were moderate producers.

Pyocyanin assays

For the detection of pyocyanin king A agar was used. Out of thirty samples, 12(40%) isolates show blue color pyocyanin production.

Twitching motility

For checking twitching motility of isolates LB agar was used. Out of thirty samples most isolates showed twitching motility and about 90% isolates were positive for twitch motility.

Influence of clove oil on QS mediated virulence factors

When the inhibitory influence of half of MIC of clove extract (12.8%) was detected on QS dependent virulence factors like pyocyanin expression, biofilm and twitching motility, it was found that all these QS mediated virulence factors were inhibited by clove oil completely in all tested isolates. Influence of clove oil was measured at multiple sub minimum inhibitory concentration on QS mediated virulence factors expression in *P. aeruginosa*. The study showed a concentration-dependent reduction in all the QS examined associated phenotypes. The decrease in biofilm expression was detected in examine bacterial isolate when inoculated in the presence of (12.8%) clove oil. The decline in the expression of pyocyanin ranged from 35% to 70% at different minimum inhibitory concentrations (12.8%) of oil concentration. The motility in the presence and absence of trial concentration was evaluated through twitching motility assay. Oil-treated bacterial cells revealed reduced flagellar motility on LB agar medium. All trial concentration demonstrated considerable decline (45–85%), in contrast to control as in Figure 4.

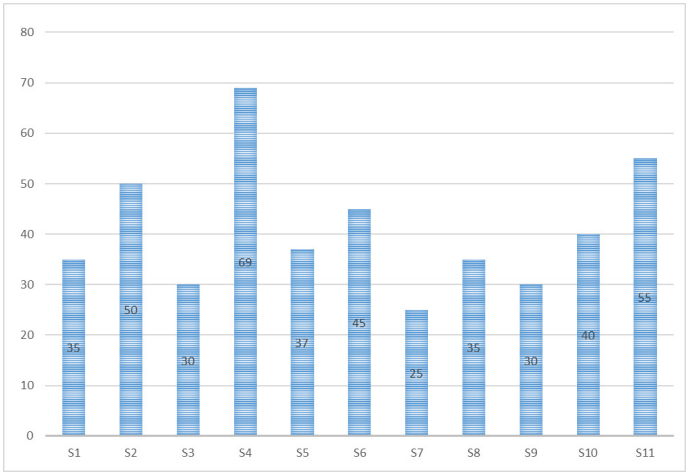

Figure 5: *The percentage of inhibitory influence of clove oil on biofilm formation in most eleven selected virulent strains.*

Four las and rhl QS system genes were found combined in 20.00% of the isolates in the current investigation. Furthermore, at least one of the four QS system genes was present in 84.00% of the isolates (Table 2). The

findings unequivocally show that *P. aeruginosa* isolates from sample taken from cancer (76.00%) and other (68.00%) have relatively high levels of the two QS systems' genes.

Discussion

Pseudomonas aeruginosa is an important nosocomial pathogen of humans worldwide (Startseva *et al.*, 2009). It causes infection in patients including those whose immune system is suppressed due to surgery. According to the USA Cystic Fibrosis organization, about 80% of people under the age of eighteen years with cystic fibrosis, patients that are using tobramycin for long term and the patients with diabetes commonly infected with multiple antibiotics resistance *P. aeruginosa*. Infection caused by this bacterium is difficult to diagnose mechanism because this bacterium developed resistance to these mechanisms. Surprisingly, these systems are commonly present instantaneously, thereby showing multi resistance phenotypes. This is compatible with our findings, where all the specimens examined exhibited great degree resistance to the maximum of the antimicrobics used (Driscoll *et al.*, 2007).

P. aeruginosa express different virulence factors that help in their pathogenesis. During their infection, this bacterium infects different tissues and some of their virulence factors play a vital role in the pathogenesis of their infection as compared to other virulence factors (Wang *et al.*, 2013). The expression of these different virulence factors in this bacterium is mediated via a cell- cell communication process termed as Quorum sensing. QS System plays a vital part in establishment of *Pseudomonas* infections (Senturk *et al.*, 2012) The significance of QS to initiate a good infection has been evaluated in various infection trials such as inflammation of cornea also called keratitis, mouse burn wounds and pulmonary infection by using QS lacking isolates. In the current research work, we explored the importance of QS mechanism in causing infection of this bacterium by revealing the presence of QS organized virulence factors like twitching motility, biofilm and pyocyanin production in 30 different clinical isolates which were obtained from wounds, sputum as well from urine and associating these with the presence of four earlier stated genes mediated by AHL molecules (Van Delden and Iglewski, 1998).

Table 2: *Primer used in this study.*

Amplified species specific	Oligonucleotide sequence 5′→3′	Product size (bp)	References
QS intact genes			
lasI F	ATGATCGTACAAATTGGTCGGC	605	Schaber *et al.*[10]
lasI R	GTCATGAAACCGCCAGTCG		
lasR F	ATGGCCTTGGTTGACGGTT	725	
lasR R	GCAAGATCAGAGAGTAATAAGACCCA		
rhlI F	CTTGGTCATGATCGAATTGCTC	625	
rhlI R	ACGGCTGACGACCTCACAC		
rhlR F	CAATGAGGAATGACGGAGGC	730	
rhlR R	CTTCAGATGAGGCCCAGC		
QS internal genes			
lasI F	TCGACGAGATGGAAATCGATG	363	Schaber *et al.*[10]
lasI R	GCTCGATGCCGATCTTCAG		
lasR F	TGCCGATTTTCTGGGAACC	362	
lasR R	CCGCCGAATATTTCCCATATG		
rhlI F	CGAATTGCTCTCTGAATCGCT	143	
rhlI R	GGCTCATGGCGACGATGTA		
rhlR F	TCGATTACTACGCCTATGGCG	207	
rhlR R	TTCCAGAGCATCCGGCTCT		
lasI F	CGTGCTCAAGTGTTCAAGG	295	Zhu *et al.* [11]
lasI R	TACAGTCGGAAAAGCCCAG		
lasR F	AAGTGGAAAATTGGAGTGGAG	139	
lasR R	RGTAGTTGCCGACGACGATGAAG		

Links Researchers

The inhibition of QS mediated virulence factors such as pyocyanin, biofilm formation, twitching motility and are the vital pathogenic objective. The hunt for a safe and better QS inhibitor agent is likely to be beneficial in curing disease caused by multiple drug resistance bacteria. As compared with traditional antibiotics, potent QS inhibitors hinder QS system and weakens virulence without affecting bacterial growth (Norizan *et al*., 2013). Multiple studies have explored the potency of different Phyto compounds and plant extracts in hindering QS mediated virulent factors in *Ps. aeruginosa*. Though the mode of mechanism through which these compounds inhibit these virulence factors are yet to be explored, but it is probable that these extracts directly or indirectly acting on inhibition of auto induced signal molecules synthesis and as result inhibit QS mechanism thus leading to inhibition of QS mediated phenotypes (Farooq *et al*., 2022).

In this present research work, trials were carried out to evaluate the influence of clove oil sub-MICs against QS mechanisms in *P. aeruginosa*. During study, when minimum inhibitory concentration of clove oil was evaluated on biofilm formation, pyocyanin making and twitching motility in eleven selected isolates, then these mentioned QS regulated virulence factors were stopped in all observed strains. This study found differences between the numbers of internal and intact genes, and the overall number of internal genes was more than the number of intact genes, which is consistent with the findings of earlier studies (Norizan *et al*., 2013; Wang *et al*., 2013).

Our results are the same as those stated by (Husain *et al*., 2003) who observed that sub-MIC of clove oil notably minimized multiple QS mediated virulence factors in clinical isolates of *Ps. aeruginosa.* Our results also suggest agreement with those of (Aboushleib *et al*., 2015) who revealed that clove oil hindered QS mediated virulence factors in *P. aeruginosa*. The existence of potent constituents showing QS inhibitory potency in the clove oil extracts may be valuable in the formulation of antimicrobials and anti-infective medications that vary from traditional antimicrobics that are bacterial killing or stop bacterial growth, as they are not supposed to disturb normal flora, nor this developed resistance.

Conclusions and Recommendations

The results of the current study confirmed that the QS have a significant role in the disease causing of *P. aeruginosa*. Therefore, compounds that weaken QS could be potential therapeutic agents. These compounds deliver alternative medication for treating emerging bacterial infections deprived of leading to antibiotic resistance as they do not cause selection pressure. Our study also exposed the anti-QS and anti-biofilm potency of clove oil against *P. aeruginosa* isolates. This potency could be utilized in formulating oil to work as an anti-pathogenic agent itself or in association with antimicrobics in treating drug-resistant bacteria. The effects of these new therapeutic approaches must be guaranteed in animal trials and clinical studies.

Novelty Statement

This study explores the role of quorum sensing (QS) genes in regulating QS-dependent virulence factors in pathogenic bacteria and investigates the in-hibitory potential of clove oil as a natural QS disruptor. The findings aim to highlight clove oil's effectiveness in attenuating bacterial virulence by targeting QS pathways.

Author's Contribution

Sadia Siddique and Muhammad Farooq: Conceptualization, writing original draft preparation and writing, review, and editing.
Sadia Siddique: Methodology.
Ifra Siddique: Formal analysis.
Muhammad Farooq: Visualization and Supervision.
All authors have read and agreed to the published version of the manuscript.

Funding
This research received no external funding.

Ethical approval
This study did not require formal ethical approval as the samples were obtained from the hospital with due permission and without any direct involvement of human participants or patient interventions. All procedures were conducted in accordance with institutional and ethical guidelines.

Statement of conflict of interest
The authors have declared no conflict of interest.

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